STIMULATION OF MYOCARDIAL AMP-ACTIVATED PROTEIN KINASE BY AICAR INCREASES CARDIAC GLUCOSE UPTAKE AND CAUSES GLUT4 AND GLUT1 TRANSLOCATION IN VIVO

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Stimulation of Myocardial AMP-activated Protein Kinase by AICAR Increases Cardiac Glucose Uptake and Causes GLUT4 and GLUT1 Translocation In Vivo

by
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Class of 1999

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I. ABSTRACT

Ischemia stimulates heart GLUT4 and GLUT1 translocation and glucose uptake in vivo. Stimulation of AMP-activated protein kinase (AMPK), an enzyme which mediates many of the effects of ischemia and which has been described as a cellular "fuel gauge," with 5-aminoimidazole-4-carboxamide-1-ribofuranoside (AICAR) increases 2-deoxyglucose uptake and causes GLUT4 translocation in isolated cardiac papillary muscles. AMPK activation may also increase heart FFA uptake in vitro. In order to study the effects of specific cardiac AMPK activation on GLUT translocation and heart substrate metabolism in vivo, we selectively infused AICAR (12.5mg/min x 90 min) intra-arterially into the anterior, LAD region of the heart in 8 anesthetized dogs. AICAR increased myocardial AMPK activity 3-fold in the anterior region compared to the posterior control region. AICAR increased the myocardial uptakes of glucose (2.3 fold) and free fatty acids (1.4 fold), but did not alter heart lactate or oxygen uptake. AICAR did not alter the energetics of the myocardium and the myocardial concentrations of ATP, AMP, ADP and creatine phosphate, which normally regulate AMPK activity, did not change. Cell fractionation and subsequent immunoblotting of purified membrane fractions demonstrated AICAR-induced translocation of both GLUT4 (1.8 fold, $p<0.002$) and GLUT1 (1.7 fold, $p<0.001$) from intracellular membranes in sarcolemma. Therefore, AMPK activation by regional AICAR infusion increased myocardial glucose uptake and caused translocation of both GLUT1 and GLUT4 in vivo without changing cellular energetics. AICAR also modestly increased heart FFA uptake consistent with the known effect of AMPK to inhibit acetyl CoA carboxylase. These findings support the

hypothesis that ischemia-induced increases in heart glucose uptake and glucose transporter translocation may be mediated by activation of AMPK.

II. INTRODUCTION

Myocardial Glucose Metabolism

Cardiovascular disease, largely in the form of ischemic heart disease, is the leading cause of death in the developed world and in the next two generations will also become the leading cause of mortality in the third world. [Organization, 1998 #1] The past few decades have brought significant advances in the diagnosis and treatment of ischemic heart disease, both acutely during an episode of myocardial ischemia and chronically in optimizing cardiac function in the diseased heart and preventing further episodes of ischemia or cardiac death. Cholesterol control, hypertension management, procedures to revascularize ischemic myocardium, a wide array of pharmacological agents for hemodynamic control and other measures have given clinicians many tools to control ischemic heart disease. Recently, the understanding that clot formation plays a central role in the sudden loss of perfusion in myocardial ischemia has also led to the development of various thrombolytic therapies to quickly reperfuse myocardium. The observation that the revascularized, optimally loaded heart displays continued dysfunction following thrombolysis even while it remains viable has highlighted the role of metabolic derangement in myocardial ischemia and renewed interest in the metabolic changes that accompany ischemia and their contribution to contractile dysfunction. [Taegtmeyer, 1995 #3]

Like all muscle, the heart functions to convert chemical energy into mechanical energy for pumping blood. The energy for this comes from metabolic substrate

oxidation which requires oxygenated blood and sufficient metabolite delivery. While the heart comprises just 0.5% of body weight it receives 4% of cardiac output and accounts for 10% of oxygen consumption. To meet its energy requirements the 300g heart uses a large amount of energy producing 35kg of ATP per day. [Willerson, 1995 #2] The myocardium uses free fatty acids (FFA) and glucose for substrate during aerobic metabolism. The choice of substrate depends on arterial concentrations and is also influenced by hormones, workload and oxygen availability. [Willerson, 1995 #2] This leads to a preference for FFA during fasting, glucose after meals and lactate and FFA during exercise. The myocardium can also use ketone bodies and even amino acids to produce energy and under certain conditions also uses endogenous energy sources such as glycogen and triglycerides. [Taegtmeyer, 1995 #3]

Glucose metabolism in myocardium, as in all tissues has two main components, glycolysis and glucose oxidation. Glycolysis can produce ATP without oxygen and while it usually contributes less than 10% of the ATP in non-ischemic conditions, it has a special role in maintaining the ion potential in the cell membrane. [Oliver, 1994 #4] Glucose is converted to pyruvate during glycolysis and once inside mitochondria, pyruvate dehydrogenase produces acetyl CoA which enters the TCA cycle generating reducing equivalents which are oxidized to produce ATP and water. Glucose can also be converted to glycogen under regulation of glycogen synthase or enter the pentose phosphate shunt to produce NADPH and ribose. Oxidation of fatty acids is the other main source of acetyl CoA production in mitochondria but is slightly less efficient than glucose oxidation requiring a greater amount of oxygen to produce each ATP.

During myocardial ishcemia, there is compromised tissue perfusion and oxygen delivery. However, residual anterograde perfusion and collateral blood flow maintains some residual availability of oxygen and substrate. [Willerson, 1995 #2] Anaerobic glycolysis becomes important in the production of a limited amount of ATP which along with residual glucose oxidation may or may not be capable of maintaining function. There is a shift away from aerobic fatty acid oxidation and an accumulation of fatty acids. [Lopaschuk, 1998 #5] In severe ischemia, catecholamines also stimulate lipolysis in adipocytes and contribute to a rise in fatty acids. [Goodwin, 1994 #8] This elevation in fatty acids may be arrythmogenic and contribute to depressed cardiac function. [Lopaschuk, 1998 #5; Goodwin, 1994 #8] Glycogen stores, are also depleted following severe ischemia. In addition, TCA intermediates are depleted when lactate, alanine and succinate accumulate and glutamate disappears. [Taegtmeyer, 1995 #3]

Facilitative Glucose Transporters

The greater utilization of glucose requires higher rates of glucose uptake. Enhanced uptake is accomplished through greater transport of glucose through facilitative glucose transporters. The family of facilitative glucose transporters (GLUTs) are passive carriers which transport glucose in an energy-independent system that moves down a concentration gradient. [Muekler, 1994 #9] Unlike the Na^+/glucose transport in intestine and kidney, the reaction is thermodynamically downhill and requires no energy. GLUTs mediate the exchange of glucose between the blood and cytoplasm and drive the balance of glucose concentrations between blood, the interstitial space and the cells. Once inside the cell, glucose is phosphorylated by hexokinase and in the absence of glucose-6-phosphatase in muscle, it remains within the cell.

Seven mammalian glucose transporter isoforms have been identified. They have 12 membrane spanning helices with about half of the amino acid residues within the phospholipid bilayer of cellular membranes. [Stephens, 1995 #10] They exhibit tissue specificity and developmental related expression and in the adult mammalian heart, GLUT4 and GLUT1 are the primary isoforms expressed. [Fischer, 1997 #21] In heart and skeletal muscle, GLUT1 mRNA and protein decrease during development while GLUT4 show increases. [Wang, 1991 #12; Santalucia, 1992 #13] GLUT1 is found primarily in cardiac myocytes of the ventricle and it controls basal glucose utilization when insulin concentrations are low and there is normal work demand. Studies in brain show that the K_m of GLUT1 is 1mM and thus GLUT1 has transport activity at normal glucose concentrations of approximately 4-8mM. [Stryer, 1995 #84; Holloszy, 1996 #19] GLUT4 has a K_m of 5mM and while it also has some basal activity its key role is to respond to external stimuli. It therefore plays a greater role in effecting changes in glucose uptake.

Kinetic studies have shown that insulin and other stimuli such as ischemia increase the V_{max} of glucose transport. [Cushman, 1980 #11] This increase in V_{max} can be caused by either an increase rate of turnover or by a greater availability of transporters at the cellular membrane. Membrane fractionation studies have established that most of the enhanced uptake is due to a shift of glucose transporters from intracellular pools where they are inactive to the sarcolemma where they can facilitate glucose uptake[Stephens, 1995 #10] Unlike other insulin sensitive tissue such as adipocytes and skeletal muscle, glucose uptake in myocardium is stimulated by translocation of both GLUT4 and GLUT1 from intracellular pools to the sarcolemma in response to insulin and ischemia.

[Young, 1995 #79] However, the degree of translocation is greater for GLUT4. Translocation of glucose transporters to the T-tubule system also enhances uptake in muscle and is important in stimulating glucose uptake. [Dombrowski, 1996 #22; Roy, 1996 #23]

The AMP-activated Protein Kinase (AMPK)

Myocardial ischemia causes an increase in glucose uptake through the translocation of the facilitative glucose transporters GLUT4 and GLUT1 from an intracellular pool to the sarcolemma through an undetermined mechanism. In addition, it causes activation of the AMP activated protein kinase (AMPK) [Kudo, 1995 #36; Kudo, 1996 #24]. AMPK was first discovered over two decades ago and is highly preserved in all eukaryotes including mammals, fungi and plants. [Hardie, 1997 #28] AMPK has been proposed to play a central protein in the cellular response to changing metabolic conditions All mammals express a similar form of AMPK while in fungi and plants AMPK homologues such as SNP, a kinase involved in the regulation of cellular metabolism, are expressed. [Mitchelhill, 1994 #33] [Carling, 1989 #31] Unlike most kinases, AMPK serves not as a signal of extracellular messengers but rather as a way for the cell to respond to environmental stress causing a compromise of energetic and metabolic conditions within the cell. It has been described as a "fuel gauge" or a "low fuel warning system" for the cell signaling low levels of ATP and high energy phosphates caused by stress. [Hardie, 1994 #25] Stresses such as heat shock, hypoxia or oxidative stress impair the ability of mitochondria to rephosphorylate ADP to ATP causing an increase in the ADP to ATP ratio which in turn activates AMPK as AMP level rise. This causes an increase in the ADP to ATP ratio and an even larger increase in

the AMP to ATP ratio through the action of adenylate kinase which catalyses the conversion of ADP into AMP and ATP. The rise in AMP levels activates AMPK and inactivates regulatory enzymes in biosynthetic pathways and increases flux through catabolic pathways thereby preserving ATP for short term needs. [Corton, 1995 #26]

Studies of purified enzyme show that AMPK is highly specific for AMP and the enzyme can only be activated by AMP or its analogs. AMP affects the AMPK system in four distinct ways (figure 1) [Hardie, 1997 #28; Corton, 1995 #26] First, it binds AMPK and causes direct allosteric activation. When the enzyme is in a phosphorylated state, allosteric activation by AMP causes a five fold increase in its activity. Second, AMP binds to dephosphorylated AMPK and makes it a better substrate for AMPK kinase (AMPKK). Phosphorylated AMPK has about a 50-fold greater activity than non-phosphorylated AMPK. Third, it binds to phosphorylated AMPK and makes it a worse substrate for protein phosphatase 2C (PP2C), an enzyme which is responsible for almost all the phosphatase activity of AMPK. Finally, AMP binds to and activates AMPKK.

However, it is likely that AMPK activity is not determined by AMP alone. ATP levels are known to independently influence the activity of AMPK. [Hardie, 1997 #28; Corton, 1995 #26; Davies, 1989 #7] In muscle, the creatine kinase-phosphocreatine (Cr-PCr) system acts as an energy buffer to provide ATP during contraction and maintains the ATP:ADP ratio within myocytes. Recently, a decrease in the PCr:Cr ratio has been shown to increase AMPK activity in skeletal muscle. [Ponticos, 1998 #27] This is consistent with the role of AMPK in mediating a response to changes in energy state and to the important role of the creatine system in the energetics of muscle. It is likely that

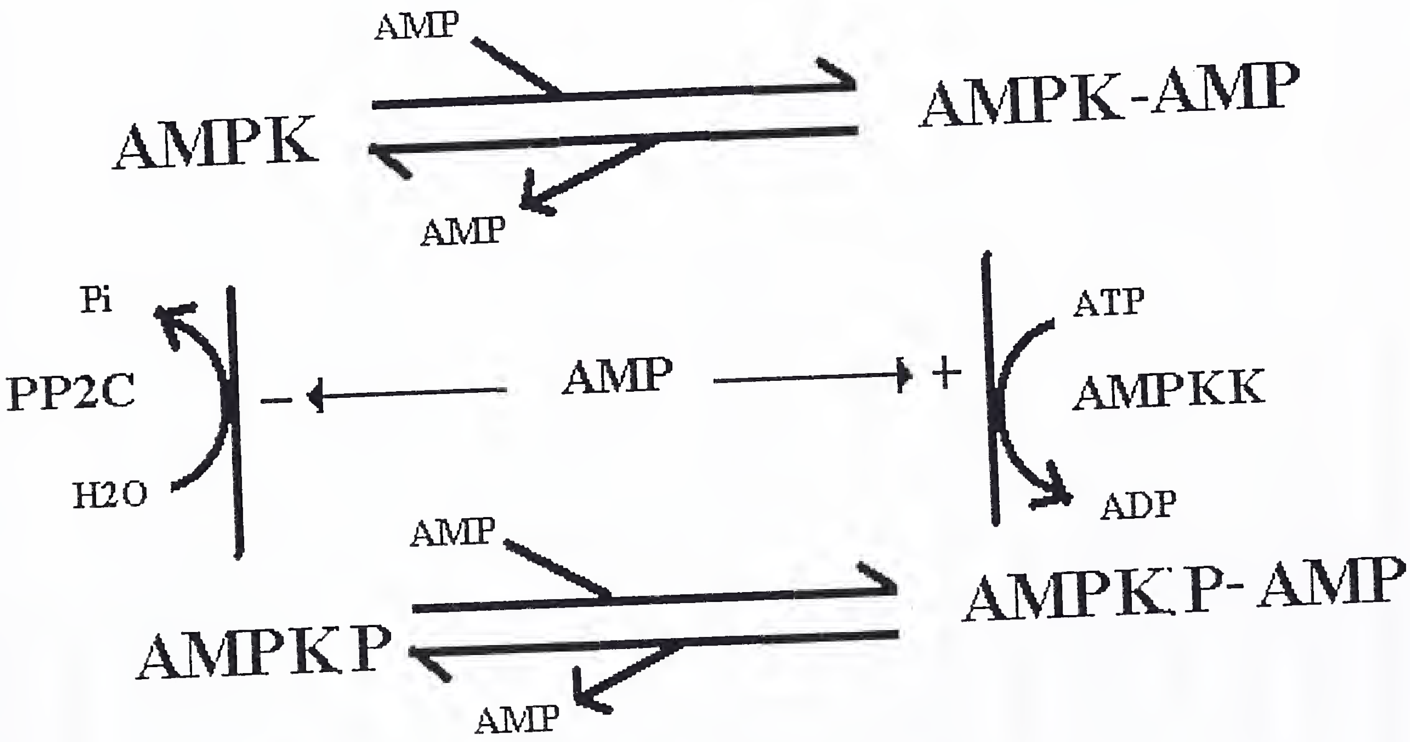


Figure 1. Model for Regulation of AMPK by AMP. AMPK exists in four states free and AMP bound, both phosphorylated and unphosphorylated. AMP promotes change to bound, phosphorylated state by binding free AMPK, making AMPKP a worse substrate for PP2C, by activation of AMPKK and by making AMPK a better

this observation can be extended to cardiac muscle as well, given the similarity of the creatine system in cardiac muscle.

AMPK was initially isolated from the liver as a kinase causing the phosphorylative deactivation of HMG-CoA reductase, the regulatory enzyme of sterol synthesis, and inhibiting sterol synthesis (and was dubbed HMG-CoA reductase kinase). [Ingebristen, 1978 #29] Subsequently, it was found to phosphorylate and inhibit acetyl CoA carboxylase and decrease malonyl-CoA. [Carling, 1987 #30] This decreases fatty acid synthesis while stimulating fatty acid oxidation by increasing carnitine-palmitoyl transferase 1 (CPT1) and promoting entry of fatty acids into mitochondria. (figure 2) In the metabolism of glycogen, AMPK inhibits glycogen synthase and stimulates glycogen phosphorylase decreasing synthesis and stimulating breakdown. [Vincent, 1996 #45] The primary role of AMPK was therefore believed to be in the regulation of these synthetic pathways either through feedback or hormonal mechanisms. However, none of the products of these pathways has been shown to cause changes in it activity and it is not subject to hormonal regulation. These actions of AMPK should therefore be viewed in light of its role in responding to changing cellular metabolic conditions and it function in increasing flux through energy producing catabolic pathways while decreasing activity through energy consuming anabolic pathways. Further study is likely to reveal more roles for AMPK in the energy metabolism of the cell.

The hypothesis that AMPK protects cells against stresses that deplete ATP suggests that it may be involved in many other processes. Studies in yeast have provided insight into other potential roles of AMPK. [Hardie, 1994 #25; Hardie, 1997 #28] Mutations in the mammalian AMPK homologue SNF1 and SNF4 genes of yeast show

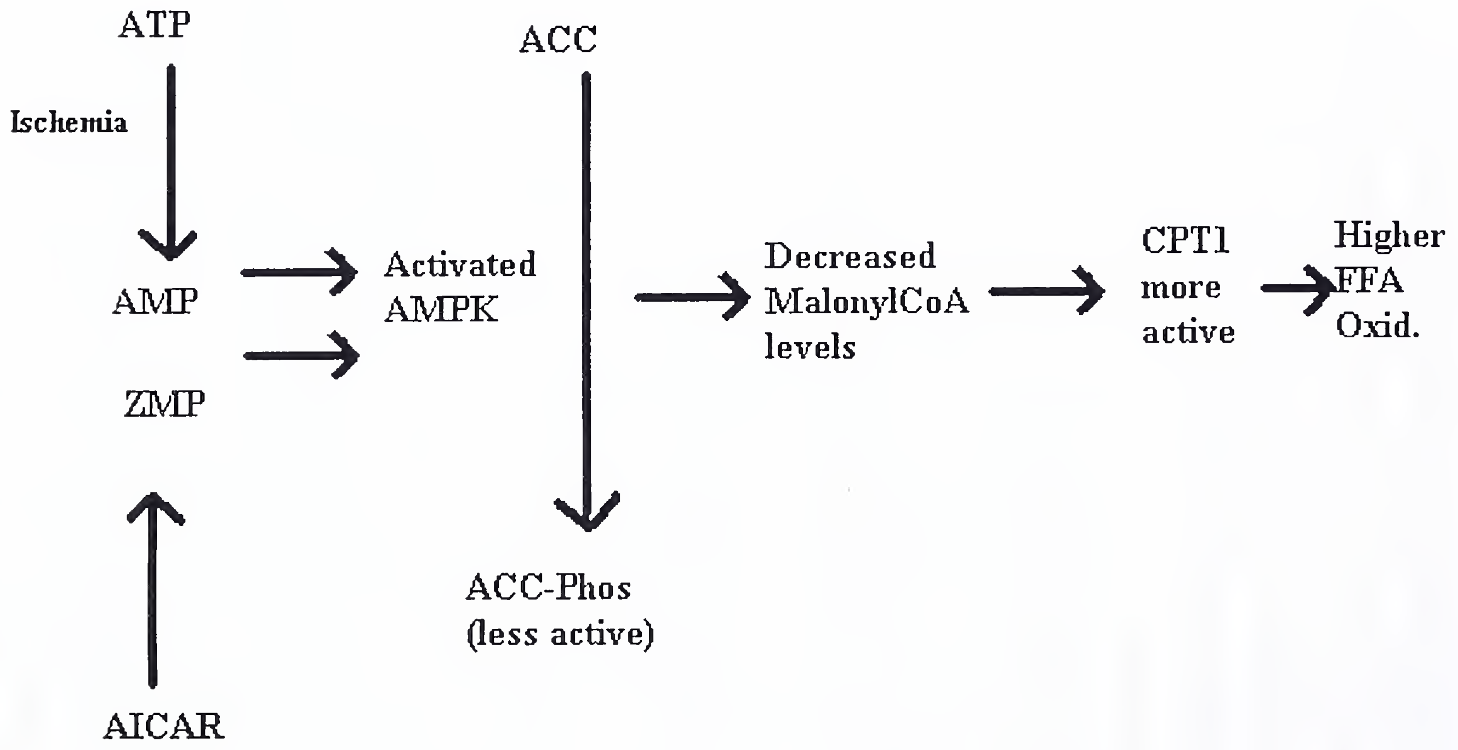


Figure 2 . Schema showing the proposed mechanism by which AMPK activation by ischemia or AICAR causes the phosphorylative inhibition of ACC which leads to a decrease in Malonyl CoA levels and increased CPT1 activity leading to higher rates of FFA oxidation.

that they are necessary for derepression of cytochrome oxidase genes required for oxidative metabolism and for expression of genes necessary for growth in the absence of glucose. In addition, they cause the phosphorylated inactivation of yeast acetyl-CoA carboxylase and are involved in cell cycle regulation. [Mitchelhill, 1994 #33] These findings suggest that the AMPK system is highly conserved and also plays a role in gene expression. Recently, the initial demonstration of AMPK activation of a mammalian gene has been made in hepatocytes where AMPK activation caused repression of the rat fatty acid synthase gene. [Foretz, 1998 #78]

AMPK has three subunits named α (63kD), β, and δ. (Davies) The α subunit is the catalytic subunit and shows a clear similarity to the SNF1 gene of the yeast saccharomyces cervisiae and other SNF1 related gene products from plants. It contains the site (Thr 172) for phosphorylation by AMPKK. [Hardie, 1997 #28] Two homologous isoforms of the α subunit, α1 and α2 have been identified. Recent studies have shown that the α2 variety has greater activity and is found in the highest levels in liver and skeletal and cardiac muscle. [Stapleton, 1996 #38] The specific distribution of the two may play a role in modulating the AMPK activity and thus, the tissue response to ischemia or other environmental stresses.

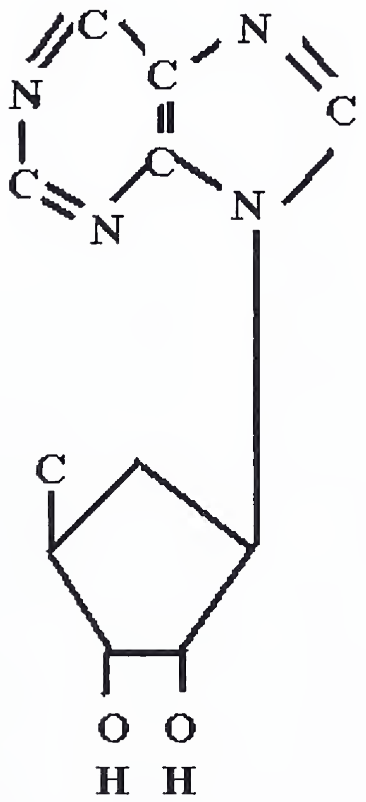
Potential Role of AMPK Activation in Glucose Transport

Two findings suggest that AMPK may be mediate the effects of myocardial ishcemia on glucose metabolism. First, AMP levels are known to rise during exercise in skeletal muscle [Hutber, 1997 #39] and in ischemic myocardium [Reibel, 1978 #40] and cause activation of AMPK in both skeletal muscle during exercise/contraction [Hutber, 1997 #39] and myocardial ischemia. [Kudo, 1995 #36] Rising AMP levels are also

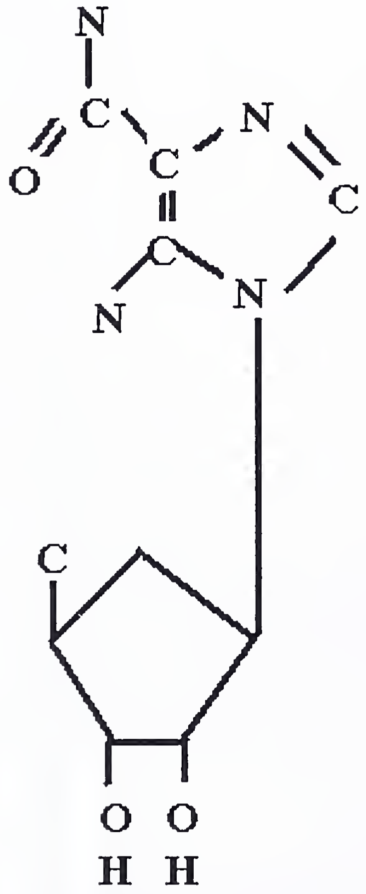
accompanied by lower concentrations of phosphocreatine. Both high AMP and lower phosphocreatine levels are known to activate AMPK. Second, ischemia/exercise induced increase in glucose uptake and GLUT 1 and GLUT 4 translocation is mediated through a pathway that is distinct from the PI3 kinase dependent translocation found with insulin. [Egbert, 1997 #41; Lund, 1995 #43] AICAR, a pharmacological activator of AMPK, also induced increase in glucose uptake that are independent of the PI3K pathway[Russell, 1998 #44; Hayashi, 1998 #50] and like ischemia are additive to insulin driven increases in glucose uptake [Russell, 1998 #44; Hayashi, 1998 #50]

AICAR is a structural analog of adenosine (figure 3). It is taken up by cells and monophosphorylated by adenosine kinase to form AICARibotide or ZMP. ZMP activates AMPK mimicking the effects of AMP on AMPK without affecting the stores of ATP, ADP, and AMP and the relevant ratios that normally control AMPK activity [Henin, 1996 #48]. The activated AMPK has similar physiologic effects as when AMP levels rise including inhibition of ACC and decrease in malonyl-CoA [Merrill, 1997 #51] and increased fatty acid oxidation.

Recently, AICAR has been shown to increase glucose uptake in perfused hindlimb muscles. [Merrill, 1997 #51] This laboratory has also demonstrated that incubation of isolated papillary muscles in AICAR causes enhancement of glucose uptake and translocation of GLUT4 glucose transporter [Russell, 1998 #44] These findings were limited by the in vitro nature of the study which allowed for the observation of the effects of AICAR on quiescent myocardial tissue not performing work. There was also no substrate competition and the uptake of deoxyglucose which does not enter any of the glucose metabolic pathways following phosphorylation was used to



Adenosine



AICAR

Figure 3

estimate glucose uptake. Evidence of translocation was limited because it was only seen in qualitative immunoflorescence studies and in poor quality membrane fractions (only 5-6 fold enrichment of sarcolemmal membranes) [Russell, 1998 #44]. The findings from these membrane studies were further limited by the increase systemic lactate concentrations caused by the systemic AICAR infusion.

In order to study the in vivo effects of AMPK activation by AICAR on glucose metabolism in vivo we infused AICAR in a selected region of the heart in dogs. This model offers several advantages. First, the direct infusion of AICAR into the coronary arteries allows a higher dose of AICAR and therefore greater activation of AMPK in myocardium without the systemic effects of high AICAR concentrations such as a buildup of lactate. This will enable a more clear interpretation of the effects of AMPK activation without the concern for the confounding effects of significant changes in substrate concentrations. Second, the large scale of the experiment allows for direct measurement of myocardial glucose uptake. Third, sufficient tissue can be obtained for membrane fractionation to study effects on glucose transporters and the use of a canine model also provides highly enriched membrane fractions. [Young, 1995 #79] Finally, physiologic parameters can also be closely followed.

III. METHODS

Animal Preparation and Experimental Protocol

Experiments were performed on fasting male mongrel dogs (40-60 lbs) according to a protocol approved by the Yale Animal Care and Use Committee. The animals (n=8) were anesthetized with sodium thiopental (70 mg/kg), intubated, and ventilated with halothane (1% to 2%), nitrous oxide (70%), and oxygen (30%) to maintain adequate anesthesia and oxygenation throughout the procedure.

As previously described, [Shi, 1995 #16; Edwards, 1992 #15] the heart was exposed through a left lateral thoracotomy. Polyethylene catheters with 25 gauge needle ends were placed in the proximal left anterior descending coronary artery (LAD) for intracoronary infusion and in the cardiac veins draining the left circumflex coronary artery (LCX) regions and LAD for venous blood sampling. [Shi, 1995 #16] Doppler crystals were sutured to the epicardial surface to measure myocardial thickening within the LAD and LCX regions using fine suturing material. A micromanometer catheter was placed to measure left ventricular pressure and dP/dt. A left atrial catheter was placed for injection of microspheres to measure coronary blood flow. Core temperature was maintained with heating pads and covering sheets.

After animal preparation, four baseline samples of arterial and LAD and LCX cardiac venous blood (4-5ml) were made over 20 minutes. Microspheres were then injected in order to measure blood flow. [Shi, 1995 #16; Edwards, 1992 #15] Small quantities of different isotopes were used for each time period. The microspheres do not affect flow or subsequent myocardial function. Hemodynamic parameters including heart

rate, aortic and left ventricular (LV) blood pressure, LV dP/dt, and cardiac output were recorded. Hemodynamic measures were recorded with data acquisition software (Dataflow, Crystal Biotech). AICAR was then infused into the proximal LAD at a rate of 12.5mg/min. (0.25ml/min) Repeat metabolic and hemodyanamic measurements were made at 30-45 minutes and at 75-90 minutes after the start of the AICAR infusion. The heart was then excised and samples (~0.5g) of tissue were taken from the central LAD and LCX regions and frozen in liquid nitrogen for AMPK activity determination. The remainder of the left ventricle was then placed in ice cold saline and utilized for membrane preparation and immunofluorescence as described below.

Analytic Measurements

Arterial and selected anterior and posterior cardiac venous blood was centrifuged and analyzed for plasma glucose and lactate concentrations with a glucose/lactate analyzer (Yellow Springs Instruments). Oxygen content in whole blood was measured with a hemoximeter (OSM 3, Radiometer America, Inc.). Plasma FFA was measured with a spectrophotometric assay (Waco) and AICAR concentrations were determined using a spectrophotometric assay which uses a diazotization coupling technique with N-(1-naphthyl)ethylenediamid. [Fujitaki, 1994 #42] The assay is adopted from established methods for the detection of primary aromatic amides and correlates to high degree (correlation coefficient of 0.98) with HPLC measurements and has a good sensitivity down to a concentration of 0.25μg/ml.

Myocardial AMP, ZMP, ADP and ATP concentrations in tissues were measured by HPLC according to a previously utilized protocol. [Sabina, 1982 #52] Approximately 100mg of frozen tissue was pulverized and homogenized in 4 times volume of 6%

perchloric, centrifuged at 3000g for 10 minutes at 4° C and neutralized with 3 M K HCO_3. Samples were then filtered and diluted 1:10 before running on HPLC system (with Systom Gold software) eluting buffers of 5mM $NH_4H_2PO_4$, pH 2.8 initially and changing to 750mM $NH_4H_2PO_4$, pH 3.9 beginning at 14 min period of 35 minute run and increasing linearly. Desired peaks were identified and quantified by comparison with standards.

Myocardial glycogen concentrations were determined using a method used by Walass et al. [Walaas, 1950 #46] Briefly, 20-40mg of tissue were solubilized in 200µL 30%KOH at 70° C and glycogen was precipitated overnight by the addition of 100µL of 6% Na_2SO_4 and 700µL of methanol. After centrifugation, the pellet was washed with methanol and resuspended in 500µL 0.5 M sodium acetate (pH 4.8) with 2mg/mL amyloglucosidase. (sigma) After incubation for 90 min at 40° C the glucose concentration was determined.

Myocardial glucose phosphate levels were measured using a spectrophotometric assay. [Lang, 1974 #85] Tissue extracts were mixed with 0.2M Tris, pH 7.5, 0.1M $MgCl_2$ and 1% NADP and absorbance was measured both prior to and following the addition of the enzyme glucose-6-phosphate dehydrogenase (1µg/µl). Various of concentrations of glucose-6-phosphate were assayed to estimate a standard curve. Citrate concentrations were measured using a spectrophotometric assay (Boerhinger Mannheim). PCA extracts were treated with citrate lyase, malate dehydrogenase, and lactate dehydrogenase which converted citrate to oxaloacetate and then lactate and in the process produced NAD^+ from NADH and causing a change in absorbance. This change in absorbance was used to calculate the citrate concentration. Creatine phosphate was

measured from PCA extracts in 50mM triethanolamine, pH 7.5, 13mM NADP, 0.1M $MgCl_2$, 0.5M glucose, and 21mM ADP in the presence of creatine kinase, hexokinase and G6P dehydrogenase. [Heinz, 1983 #49] This reaction converts $NADP^+$ to NADPH causing a shift in absorbance from which the creatine phosphate concentration can be calculated.

Membrane Preparation and Characterization

Left ventricular myocardium from the anterior region infused with AICAR and from the control posterior ventricular wall (approximately 25g each) was selected. The border zone between the two regions was discarded. Membrane fractions were prepared as described previously using differential and sucrose gradient centrifugation. [Russell, 1998 #20; Young, 1995 #79] In brief, crude homogenates (20%wt/vol) were prepared in $NaHCO_3$ (10mM) / NaN_3 (5mM) buffer with a polytron and centrifuged at 1200g for 10 minutes. The pellet was resuspended and rehomogenized and centrifugation was repeated. The supernatant (crude membrane fraction) was centrifuged at 190,000 g for 1 hour, resuspended in a 25% sucrose solution and loaded onto a discontinuous sucrose gradient (25%, 30%, 35% wt/vol) and centrifuged for 18 hours at 150,000g. The sarcolemma fraction was collected from the upper half of the 25% sucrose layer The intracellular membrane fraction was collected from the 30%/35% interface. The membranes were collected and diluted 4-5 fold in NaHCO3 (10mM/NaN_3 5mM) and then centrifuged at 190,000g for 1 hour. All procedures were done at 4° C. The membranes were resuspended in Tris (50mM), pH 7.4 and stored at -70° C. There was no statistically significant difference in the protein yields between AICAR and control regions. (table 1)

Table 1a. Enrichment of Membrane Fractions

Fraction	Marker	Enrichment vs. crudemembrane*
Intracellular	Ca^{++} ATPase	Control: 8.8 (+/- 2.59) AICAR: 8.0 (+/- 2.51)
Sarcolemmal	Na^{+}/K^{+} ATPase	Control: 239 (+/- 110) AICAR: 84 (+/- 14)

Values are expressed as mean +/- SEM. * AICAR vs Control not statistically significant for both fractions.

Table 1b. Protein Yield Membrane Fractions

Fraction	Yield (μg)*
Intracellular	Control: 1022 (+/- 285) AICAR: 955 (+/- 245)
Sarcolemmal	Control: 475 (+/- 57) AICAR: 490 (+/- 63)

Values are expressed as mean +/- SEM. * AICAR vs Control not statistically significant for both fractions.

Membrane protein concentration was measured with a spectrophotometric assay (Bio-Rad Laboratories) using bovine serum albumin as a standard. Enrichment of fractions for the sarcolemma and sarcoplasmic reticulum proteins, Na^+, K^+-ATPase and Ca^{++}-ATPase, respectively, was assessed by immunoblot analysis of the membrane fractions with monoclonal antibodies to the B-subunit of the Na^+, K^+-ATPase (gift from Dr. Michael Caplan) and the SERCA-2 calcium pump (Affinity Bioreageants, Inc). Immunoreactive protein was detected and quantified with ^{125}-I-antimouse IgG (Amersham Co). Sarcolemmal membranes were highly enriched for the plasma membrane marker Na^+, K^+-ATPase (80-240 fold when compared with crude fractions) while the intracellular fractions were enriched for the intracellular marker Ca^{++}-ATPase. (about 8 fold relative to crude membrane fraction). There was no statistically significant difference in these enrichments. (table 1)

GLUT 4 and GLUT 1 Immunoblot Analysis

Sarcolemma and intracellular membrane proteins (20μg) underwent SDS-PAGE on 10% and were transferred to PVDF membranes. [Young, 1995 #79] (Bio-Rad Laboratories) Membranes were blocked with 5% non-fat milk in PBS and then incubated with polyclonal GLUT4 that was partially purified in an IgG column (1:5000) or GLUT1 in whole serum (1:1000) antibodies with specific immunoreactivity in canine heart (LHY1) overnight at 4° C. The membranes were washed and incubated with ^{125}-I-protein A, washed again prior to autoradiography and then quantified using a gamma well counter (Packard Instruments).

AMPK was assayed using a previously described method which measures the phosphorylation of a purified 17 amino acid peptide containing the consensus sequence (SAMS) for AMPK. [Winder, 1996 #34] Approximately 100mg of tissue was homogenized in 5x volume of isolation buffer containing 100mM Tris-HCl, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM benzamidine, 400μg/ml trypsin inhibitor, 50mM NaF, 5mM sodium pyrophosphate, 10% v/v glycerol. Homogenates were centrifuged at 14,000g for 30 min, polyethylene glycol 8000 (PEG) was added to the supernatant to achieve a concentration of 2.5% (w/v) and then centrifuged at 10,000g for 10 min. PEG was then added to bring to a final concentration of 6% (w/v), centrifuged again at 10,000g, and the pellet was finally resuspended in 100μl of homogenization buffer. Aliquots were taken to determine protein concentration and samples were stored in liquid nitrogen and stored for a maximum of 1-3 days.

Isolated protein (2μg) was incubated in buffer containing 96mM Hepes-NaOH, pH 7.9, 192mM NaCl, 20%glycerol, 240mm SAMS, 2.4mmAMP, 5mM $MgCl_2$, 200μM ATP with 200-400 dpm/pmol ^{32}P ATP (Lopachuk 2) at 30°C for 5 minutes. Aliquots were then removed and spotted on phosphocellulose filter paper (Whatman P81), washed 4 times with 150mM phosphoric acid over 30 minutes and once in acetone for 20 minutes, dried and counted with liquid scintillation fluid . AMPK activity was determined after subtaraction of background activity.

Calculations and Statistical Analysis

Myocardial blood flow was calculated according to the following equation:

$$\text{Blood flow} = (\text{dpm/mg})^{-1} * (\text{dpm/ml}) * (\text{ml}) * (\text{sec})^{-1}$$

The [(dpm/mg) is obtained from counting samples of tissue, (dpm/ml) is calculated from the ratio of known quantity of isotope to measured quantity of blood, ml is injected volume, and time is also carefully measure (45 seconds)].

The myocardial uptake of glucose, lactate, and FFA were calculated as the product of the cardiac extractions (AV concentration difference) and the myocardial plasma flow. Plasma flow was in turn calculated from the blood flow and individually hemoglobin concentrations. Myocardial oxygen consumption was calculated as the product of oxygen extraction and the blood flow. The content of glucose transporters in either sarcolemma or intracellular membrane fraction was expressed as the binding per microgram protein calculated from the density (dpm/μg protein) product of the dpm of ^{125}-I-protein A and measured protein yield of that fraction (μg/g tissue) or expressed as the relative content of transporters of each fraction)

All values are expressed as mean+/- SEM. Paired t test was used to compare AICAR and control values. A value of $p < 0.05$ was considered significant.

IV. RESULTS

Myocardial AICAR and Nucleotide Levels and AMPK Activity

During AICAR infusion in the LAD, the venous concentrations in the LAD region were 2510μM (+/- 78) as compared to 353μM (+/- 55) in the LCX region at the end of the 90 minute period (table 2) There was a wide range in concentrations, reflecting changes in flow. The medians were 1897μM in the LAD region and 178μM in the LCX region. A value of 1399μM in the LCX region (compared to 2280μM in the LAD region) in one sample, probably due to the presence of venous collaterals that were inaccessible for suturing and separating, caused the average overall LCX region AICAR concentration to be much higher. The concentration of ZMP was 321μM (+/- 69) in the AICAR region and 158 μM (+/-34) ($P < 0.05$). There was no significant difference in the concentrations of AMP, ADP, or ATP levels (Table 2).

AICAR infusion resulted in a significant 3-fold higher AMPK activity in the anterior region (73pmol/min/mg to 253pmol/min/mg) as compared to the LCX region which did not receive AICAR (figure 4).

Subcellular Membrane Distribution of GLUT-1 and GLUT-4

Analysis of western blots show that both GLUT-1 and GLUT-4 were found primarily in the intracellular compartment in regions of myocardium that did not receive AICAR. As determined by activity of the bands and yield of each fraction (see Methods), the sarcolemma contained 30% of GLUT-1 and 22% of GLUT-4. (figures 5, 6)

In the region that received AICAR, there was evidence of translocation of both glucose transporters from the intracellular pools to the sarcolemma. The relative sarcolemma

distribution of GLUT-4 increasd 2-fold to 40% (P<.002) while that of GLUT-1 increased two-thirds to 50% (p<.001). (figures 5, 6) The ratio of sarcolemmal to intracellular transporter densities increased significantly for both GLUT-1 (0.75 to 1.3, p<0.0007) and GLUT-4 (0.35 to 0.68, p< 0.009). (table 3)

Myocardial Metabolism in vivo

Arterial concentrations of FFA, lactate, and oxygen content did not change during the local intracoronary infusion of AICAR . However, there was a significant decrease in glucose concentration following AICAR infusion at 45 minutes and 90 minutes. (table 4) The systemic arterial concentrations of AICAR were 93μM at 45 minutes and 123μM at 90 minutes. These AICAR concentrations are approximately 5% of the concentrations in the LAD bed and are not known to affect glucose concentration and is there not the likely cause of the drop in glucose concentration observed. Other systemic changes may be have caused a small hypoglycemic effect. Insulin levels were not determined. The AICAR region showed a 2-fold increase in myocardial glucose uptake, extraction, and arterial-venous difference (AVD) in the AICAR (LAD) regions when compared with the control (LCX) region after 90 minutes of AICAR infusion. (table 4, figure 8) There was a modest (approximately 30%) increase in FFA uptake, extraction, and AVD after 90 minutes of infusion. There was a similar change after 45 minutes, although the AVD did not reach significance. (table 5) Prior to the administration of AICAR, the uptake, extraction, and AVD of glucose and FFA was similar in the two regions. Lactate and oxygen uptake showed no significant difference either in baseline period, at 45 minutes or at 90 minutes. (tables 6, 7) The myocardial uptake, extraction, and AVD of glucose and oxygen consumption were not significantly different in LCX (control) region or in

the LAD region following administration of AICAR in the LAD. However, there was significantly higher FFA extraction at 90 minutes of AICAR infusion when compared to baseline in the LAD region and a significantly lower lactate AVD in the LAD region at 45 minutes and at 90 minutes of infusion when compared to baseline.

Glycogen, citrate, G6P, and creatine phosphate levels were not significantly different in the AICAR and control regions.(table 2)

Hemodynamics and Myocardial Thickening

Infusion of AICAR in the LAD region had no effect on thickening fraction when compared to the control LCX region. (table 8, 9) However, there was statistically significant, 25% decrease in the thickening fraction of the LAD region following 90 minutes of AICAR infusion. (table 9) Heart rate, mean arterial pressure, dP/dt and LVEDP were unchanged during AICAR infusion when compared to baseline Following AICAR infusion, there was a trend (P = 0.06) for higher blood flow in the AICAR perfused LAD region when compared with the LCX region. (Table 9) There was no change in blood flow following 45 minutes of AICAR infusion. (Table 8).

Table 2. Myocardial Tissue Concentrations of AICAR, Nucleotides, Glycogen, G6P, Citrate, and Creatine Phosphate

	Control	AICAR (90min)
AICAR (μM)	353 (+/-55)	2511 (+/- 78) *
ZMP (μM)	158 (+/- 34)	321 (+/-69) *
AMP (μM)	28 (+/- 548)	19 (+/- 119)
ADP (μM)	1098 (+/-114)	1217 (+/- 183)
ATP (μM)	3740 (+/-194)	4350 (+/- 249)
Creatine Phosphate (mM)	9.0 (+/- 3.6)	8.4 (+/- 2.4)
G6P (μM)	254 (+/-15)	271 (+/- 19)
Citrate (μM)	535 (+/-35)	410 (+/-28)
Glycogen (μM/g)	28 (+/-5.3)	32 (+/-4.5)

Values are means +/- SEM (n=4-6). $^*P< 0.05$ vs Control.

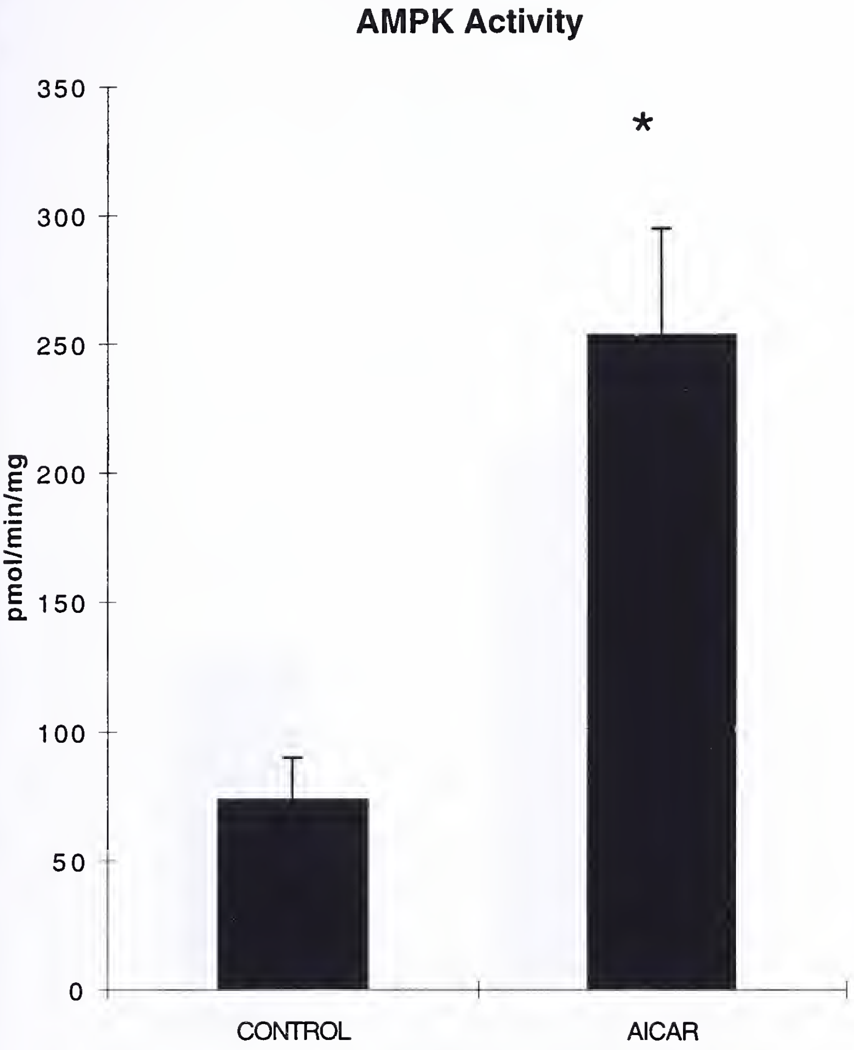


Figure 4. AMPK activity in control and AICAR infused myocardium. Values are expressed +/- SEM (n=3).
* $P < 0.05$

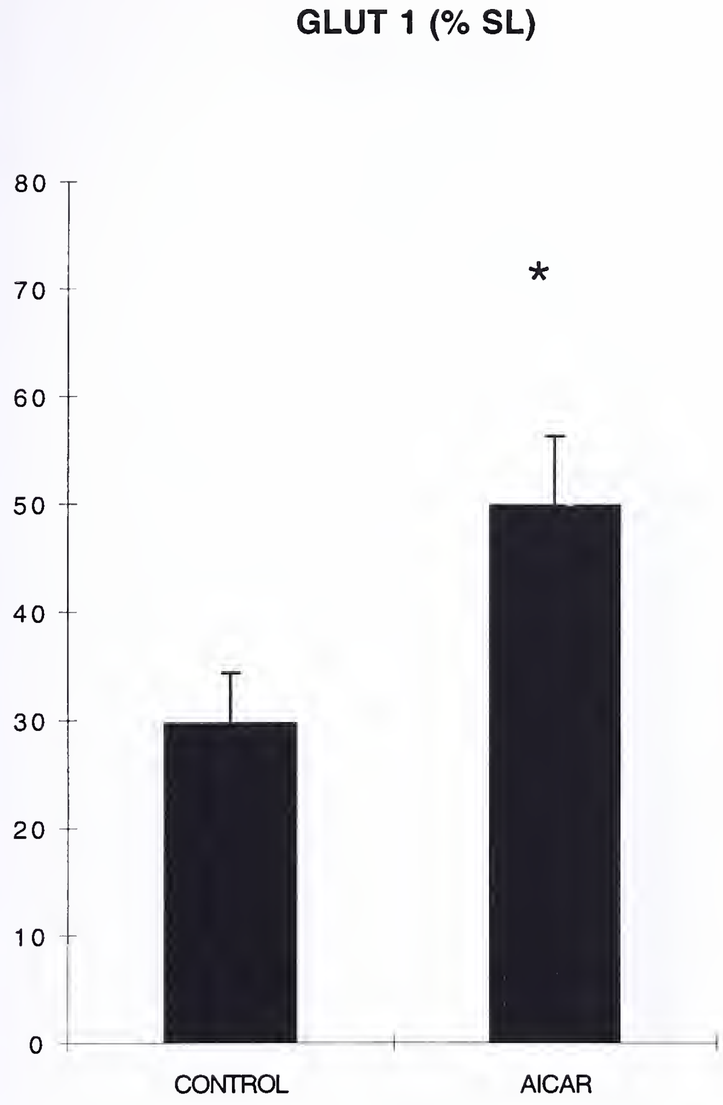


Figure 5. Percentage of GLUT1 in sarcolemma in myocardium from control and AICAR-perfused regions. Values are mean +/- SEM. * P < 0.005 vs control region.

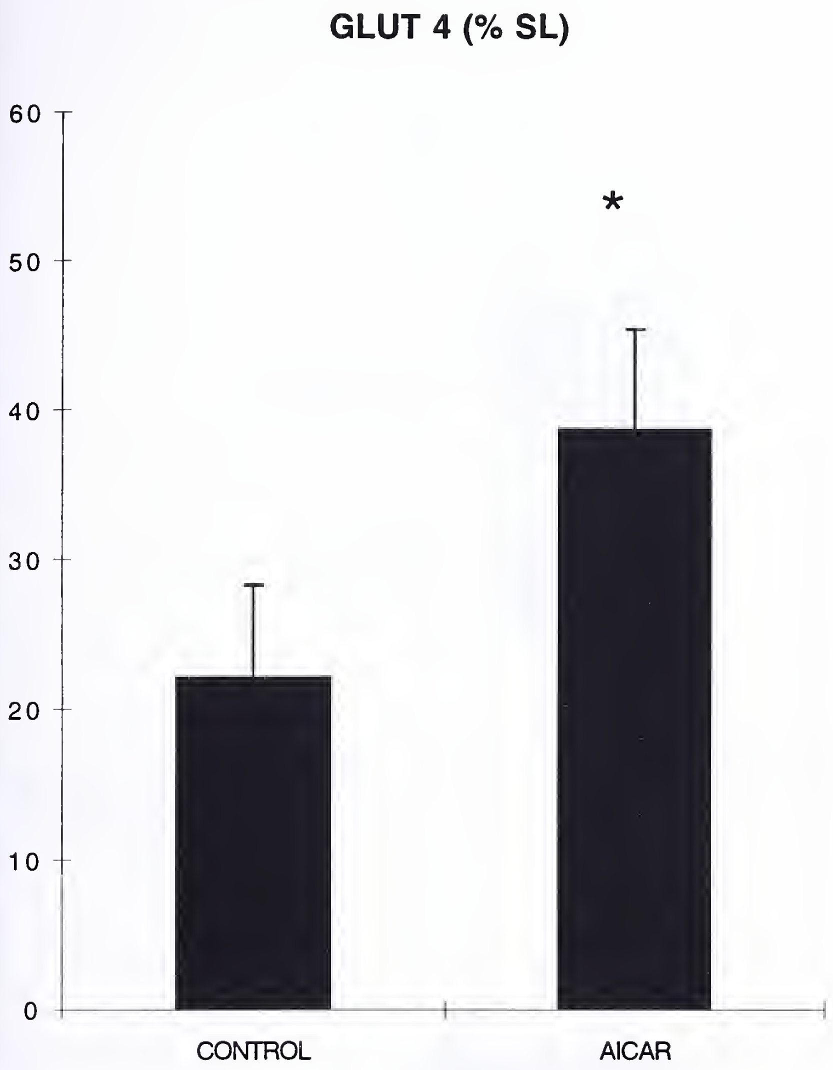


Figure 6. Percentage of GLUT4 in sarcolemma in control and AICAR perfused myocardium. Values are mean +/- SEM. * $P < 0.001$ vs control.

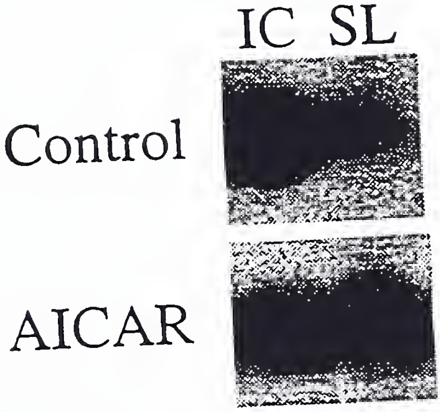


Figure 7a. GLUT4 immunoblot of intracellular and sarcolemma membranes.

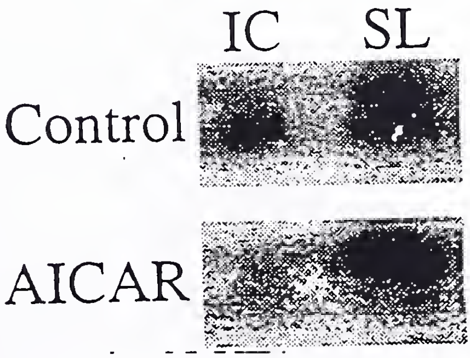


Figure 7b. GLUT1 immunoblot of intracellar and sarcolemma membranes.

Table 3. Effect of AICAR on GLUT1 and GLUT4 Distribution in Intracellular (IC) and Sarcolemmal (SC) Fractions

	Control	AICAR
GLUT1 IC:SL	0.7 (.12)	1.3 (0.16)*
GLUT1 % SL	30 (6.4)	50 (4.7)*
GLUT4 IC:SL	0.4 (0.09)	0.8 (0.05)*
GLUT4 % SL	22 (6.1)	39 (6.6)*

Values are expressed as mean +/- SEM. * $P< 0.005$ vs Control.

Table 4 Effect of AICAR on Arterial Concentration, Arterial-Venous Difference,Extraction and Uptake of Glucose.

	Arterial	A-V Difference	
		LAD	LCX
Baseline	6.02 (0.24)	3.46 (0.067)	2.83 (0.058)
45 min	5.52 (0.23)**	3.81 (0.052)	3.54 (0.054)
90 min	5.37 (0.23)**	3.95 (0.059)*	2.09 (0.043)

Extraction:	LAD	LCX
Baseline	3.2 (1.01)	2.7 (0.96)
45 minutes	3.8 (0.91)	3.7 (1.06)
90 minutes	4.09 (1.08)*	2.2 (0.87)

Uptake:	LAD	LCX
Baseline	13.1 (4.3)	10.5 (3.8)
45 minutes	13.5 (3.4)	9.9 (2.6)
90 minutes	13.6 (3.3)*	5.9 (2.1)

Arterial concentration and A-V difference in mM. Extraction is in %/100. Uptake is in µmol/100g/min. . Values are expressed as mean +/- (SEM). * P< 0.005 vs Control/ LCX. **P<0.05 vs Baseline.

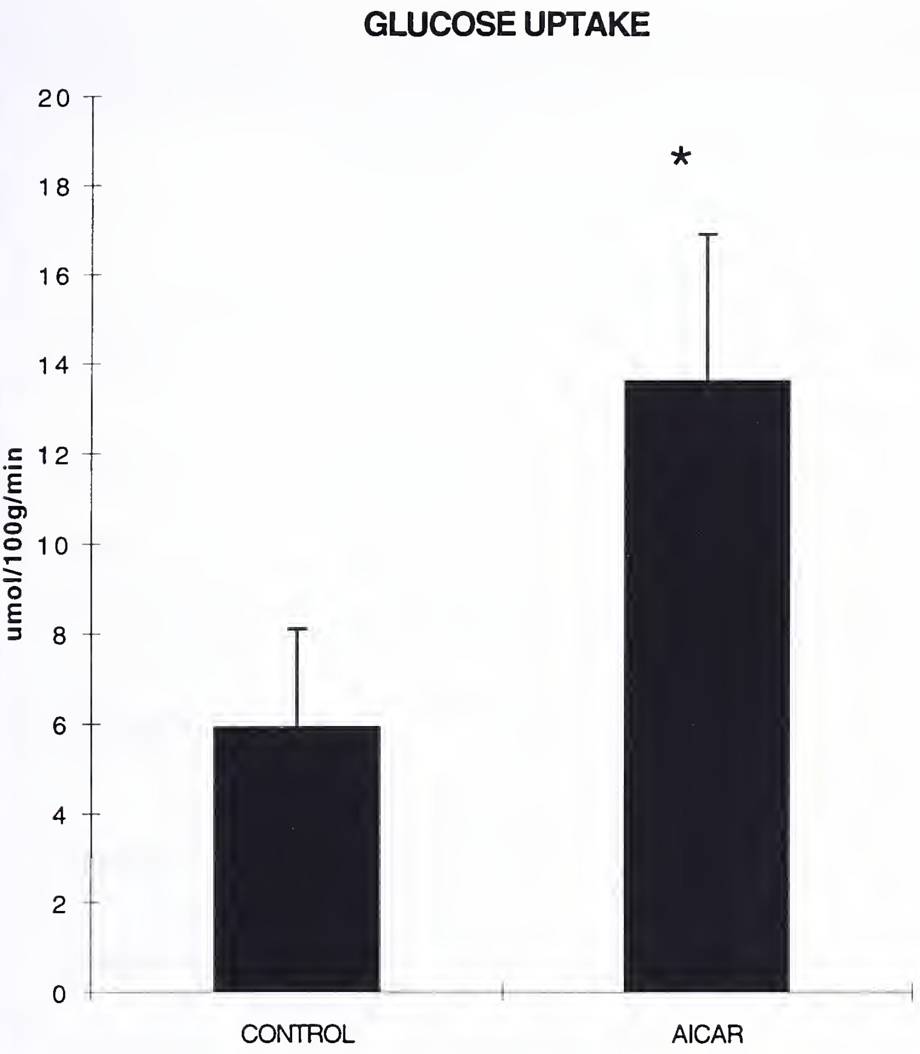


Figure 8. Uptake of glucose in myocardium from control and AICAR infused regions. Data are quantified as umol/100g/min. Values are mean +/- SEM (n=8). *P< 0.05

Table 5 Effect of AICAR on Arterial Concentration, Arterial-Venous Difference, Extraction and Uptake of FFA.

	Arterial	A-V Difference	
		LAD	LCX
Baseline	635 (106)	174 (41)	163 (46)
45 min	527 (60)	193 (42)	164 (32)
90 min	507 (43)	207 (25)*	176 (26)

Extraction:	LAD	LCX
Baseline	27 (4.4)	22 (3.0)
45 minutes	35 (4.3)	30 (3.4)
90 minutes	42 (4.2)* & **	34 (4.5)

Uptake:	LAD	LCX
Baseline	11.8 (2.5)	10.3 (2.0)
45 minutes	13.3 (2.5)	9.1 (1.1)
90 minutes	13.7 (2.0)*	10.0 (1.5)

Arterial concentration and A-V difference in µM. Extraction is in %/100. Uptake is in µmol/100g/min. . Values are expressed as mean +/- (SEM). * $P< 0.005$ vs Control. **$P<0.05$ vs Baseline.

Table 6 Effect of AICAR on Arterial Concentration, Arterial-Venous Difference, Extraction and Uptake of Lactate.

	Arterial	A-V Difference	
		LAD	LCX
Baseline	1.53	0.44 (0.074)	0.37 (0.067)
45 min	1.46	0.23 (0.062) **	0.27 (0.063)
90 min	1.62	0.24 (0.055) **	0.29 (0.065)

Extraction:	LAD	LCX
Baseline	29.4 (3.2)	26.8 (3.4)
45 minutes	15.2 (3.7)	17.9 (3.4)
90 minutes	13.9 (2.7)**	16.5 (4.1)

Uptake:	LAD	LCX
Baseline	27.5 (4.5)	23.1 (3.9)
45 minutes	16.3 (3.9)**	16.8 (3.8)**
90 minutes	12.3 (3.2)**	13.6 (3.2)

Arterial concentration and A-V difference in mM. Extraction is in %/100. Uptake is in µmol/100g/min. . Values are expressed as mean +/- SEM. * P< 0.005 vs Control. **P<0.05 vs Baseline.

Table 7 Effect of AICAR on Arterial Oxygen Content and Oxygen Consumption.

	Arterial	Oxygen Consumption	
		LAD	LCX
Baseline	15 (1.5)	6.7 (1.5)	5.8 (0.7)
45 min	15 (0.7)	6.6 (1.4)	5.6 (0.7)
90 min	15 (0.9)	8.9 (0.8)	8.4 (0.9)

Values are in ml/100g/min. Values are expressed as mean +/- SEM. * P< 0.005 vs Control. **P<0.05 vs Baseline.

Table 8. Myocardial Contactile Function and Hemodynamics during Baseline and after 45 Minutes of AICAR Infusion

	Baseline	**AICAR**
Thickening Fraction, %		
Control Region	17 (+/- 1.6)	17(+/- 1.5)
AICAR Region	20 (+/- 0.8)	19 (+/- 0.8)
Heart Rate, bpm	113 (+/- 5.4)	112 (+/-6.4)
Mean Arterial Pressure, mm Hg	82 (+/- 3.4)	83 (+/- 3.3)
LVEDP, mm Hg	10 (+/- 1.2)	10 (+/- 1.1)
dP/dt, mm Hg/s	1297 (+/- 99)	1239 (+/- 99)
Blood Flow, ml/g/min		
Control Region	1.01 (+/-0.08)	0.97 (+/- 0.22)
AICAR Region	1.01 (+/- 0.08)	1.01 (+/- 0.17)

Values are means +/- SEM. * $P < 0.05$ vs Baseline Period. ** $P = 0.06$ vs Control Region.

Table 9. Myocardial Contactile Function and Hemodynamics during Baseline and after 90 Minutes of AICAR Infusion

	Baseline	AICAR
Thickening Fraction, %		
Control Region	17 (+/- 1.6)	16(+/- 2.7)
AICAR Region	20 (+/- 0.8)	16 (+/- 0.5)*
Heart Rate, bpm	113 (+/- 5.4)	117 (+/-6.8)
Mean Arterial Pressure, mm Hg	82 (+/- 3.4)	79 (+/- 6.2)
LVEDP, mm Hg	10 (+/- 1.2)	9 (+/- 1.3)
dP/dt, mm Hg/s	1297 (+/- 99)	1062 (+/- 97)*
Blood Flow, ml/g/min		
Control Region	1.01 (+/-0.08)	0.81 (+/- 0.14)
AICAR Region	1.01 (+/- 0.08)	1.01 (+/- 0.17)**

Values are means +/- SEM. * $P < 0.05$ vs Baseline Period. ** $P = 0.06$ vs Control Region.

V. DISCUSSION

AMPK Activation, Stimulation of Glucose Transport and Transporter Translocation

This study demonstrates two important findings. First, it shows that activation of AMPK by AICAR results in a two-fold increase in myocardial glucose uptake in vivo. Second, it demonstrates that this increase in glucose uptake is associated with translocation of heart GLUT 4 and GLUT 1 glucose transporters from an intracellular pool to the sarcolemma suggesting that the increased glucose uptake is at least in part mediated by transporter translocation. Since myocardial ischemia and hypoxic stress or contraction in skeletal muscle are also known to activate AMPK and stimulate glucose uptake through glucose transporter translocation, this study supports the hypothesis that ischemia mediated changes in glucose may also be mediated by this kinase.

AICAR, an intermediate in the purine biosynthetic pathway, is taken up by cells and phosphorylated to form ZMP which mimics the effects of intracellular AMP causing allosteric activation of AMPK and promoting phosphorylation and covalent activation of AMPK by AMPK kinase. [Corton, 1995 #26] In this study, local intracoronary infusion of AICAR achieved concentration of approximately 2mM in the venous blood draining from the infused region. AICAR was taken up by the heart and was phosphorylated to form ZMP. The myocardial ZMP concentration averaged 310μM which approximates the concentration causing the half maximal activation of AMPK in vitro. [Henin, 1996 #48] Significantly, the concentrations of the substrates which normally determine AMPK activity, AMP, ATP, and ADP as well as creatine phosphate were unchanged indicating that the changes in AMPK activity were not an indirect effect of AICAR altering the

energetics of cardiac myocytes but rather a likely direct effect through conversion to ZMP.

Our findings demonstrated a 2-3 fold increase in the myocardial glucose uptake associated with AMPK activation during local intracoronary AICAR infusion in vivo. This is comparable to the 3-4 fold increase in glucose uptake observed during low flow ischemia in the same canine model using a similar protocol and measurement techniques in our laboratory. [Young, 1995 #79] Other studies of AMPK stimulation by AICAR also found similar effects on glucose uptake. In perfused hindlimbs, AMPK stimulation by AICAR led to a several fold increase in glucose uptake. [Merrill, 1997 #51] In vivo, systemic infusion of AICAR at a rate of 7.5 mg/kg achieved an average ZMP concentration of 350μM, similar to the concentration in the AICAR perfused region in our study, and stimulated skeletal muscle glucose uptake two fold. [Bergeron, 1998 #86] In recent in vitro studies, we observed that incubation of isolated quiescent rat papillary muscles in 1mM AICAR caused a two-fold increase in deoxyglucose uptake. [Russell, 1998 #44] Thus the findings in the intact heart in this study are congruent with these earlier studies and further demonstrate the enhancement of myocardial glucose uptake in hearts performing a physical workload while also subject to the influence of circulating hormones.

This study found that AICAR infusion is associated with translocation of myocardial GLUT4 and GLUT1 from intracellular pools to the sarcolemma. This suggests that, in myocardium, AICAR stimulates glucose uptake by increasing the number of facilitative glucose transporters available at the sarcolemma. We have recently found that AICAR also stimulates translocation of GLUT4 to the sarcolemma

using immunoflorescence in quiescent rat papillary muscles. These studies were in non-working myocardium and in an artificial hormonal and substrate milieu. This the first demonstration of AICAR induced translocation of GLUT4 in vivo and the initial evidence that AMPK stimulation by AICAR causes translocation of GLUT1 as well.

AICAR caused an increase in the sarcolemmal content of GLUT4 from 22% to 40%. Exercise or skeletal muscle and myocardial ischemia also cause translocation of glucose transporters. In our previous studies, [Young, 1995 #79] low flow ischemia led to a doubling of the distribution of GLUT4 in the sarcolemma increasing from 15% to 30%. While it had been suggested that only GLUT4 translocation is involved in the response of muscle to exercise/contraction GLUT1 also plays a role in cardiac muscle. [Douen, 1990 #65; Hayashi, 1997 #17] GLUT1 is only found in the sarcolemma of adipocytes and skeletal muscle and is not thought to be “recruitable” in these tissues. However, in myocardium we have previously found that GLUT1 is responsive to ischemia though to a lesser extent than GLUT4. Low flow ischemia caused the proportion of GLUT1 in the sarcolemma to increase by 41% to 58%. This is somewhat less than the effect of AICAR and AMPK activation which increased GLUT1 sarcolemma from 30% to 50%. [Young, 1995 #79] This provides further evidence that GLUT1 plays a unique role in myocardium in that it is recruitable and responds to stimuli to increase uptake of glucose. This is likely due to the large metabolic demands of myocardium.

AMPK Activation in Muscle

AICAR infusion stimulated AMPK activity by three fold. There is evidence which suggests that AMPK activation plays a key role in the response to oxidative stress

in both skeletal and cardiac muscle whether during contraction/exercise or ischemia. The highest levels of mRNA are found in skeletal and cardiac muscle. [Verhoweven, 1995 #53] In skeletal muscle of rats, prolonged exercise led to a several fold activation of AMPK and a subsequent inactivation of ACC and repression of malonly CoA. [Winder, 1996 #34] In perfused working rat hearts subject to global ischemia, there was a large increase in AMP levels and a 2-3 fold activation of AMPK. [Kudo, 1995 #36] AMPK activity remained high even after reperfusion reflecting, perhaps the continuing effects of AMPK phosphorylation. [Kudo, 1995 #36; Kudo, 1996 #24] Many other studies have demonstrated a stimulation of AMPK in response to exercise/contraction or ischemia [Hutber, 1997 #39; Vavvas, 1997 #80]

Given that both ischemia and AICAR infusion stimulate glucose uptake and translocation of GLUT4 and GLUT1, it is intriguing to consider that ischemia induced translocation of glucose transport and is mediated through AMPK. While the mechanism through which ischemia in myocardium and exercise/contraction stimulate glucose uptake and glucose transport translocation is not known, it is characterized by several observations demonstrating that it is distinct from the well studied insulin regulated increase in glucose uptake and glucose transporter translocation. Insulin binds to the α-subunit of the insulin receptor which leads to phosphorylation of the β-subunit and increased tyrosine kinase activity. Tyrosine kinase phosporylation of the intracellular insulin receptor substrate-1 (IRS-1) then causes activation of phosphatidylinositol-3-kinase(PI3 K). (figure 9) [Holman, 1997 #54] This kinase is inhibited by wortmannin and the action of insulin in stimulating glucose uptake and translocation is also inhibited in both skeletal muscle and cardiac muscle. [Lee, 1995

#61; Russell, 1998 #44; Hayashi, 1998 #50]This is in contrast to the ischemia mediated and exercise/contraction mediated increase in glucose uptake [Russell, 1998 #44; Bergeron, 1998 #86] and translocation in skeletal muscle which is not influenced by PI3K inhibition by wortmannin. Wortmannin did not block the stimulation of glucose uptake by AICAR in skeletal muscle [Russell, 1998 #44; Bergeron, 1998 #86] and in isolated papillary muscles. [Russell, 1998 #44] A second observation which suggests a distinct pathways in insulin and ischemia/exercise stimuated glucose uptake is the additive nature of insulin and ischemia/contraction. AICAR and ischemia are not additive but AICAR and insulin are additive in skeletal muscle. [Bergeron, 1998 #86] Finally, our study bolsters the hypothesis because pharmacological AMPK stimulation was shown to cause translocation and increased transport in vivo in physiologic conditions.

Other pathways for the ischemia mediated glucose transport have been hypothesized. Due to the role of PI3K in insulin mediated on glucose, a potential role for the kinase in ischemia/contraction was hypothesized in muscle. However, studies of PI3K inhibition suggest that it is not a mediator in ishcemia/contraction. [Egbert, 1997 #41; Lund, 1995 #43]. Protein kinase B (Akt) has been identified as a downstream target for PI3K in the insulin mediated GLUT4 translocation and it may serve as a target for AMPK as well. [Hayashi, 1997 #17] Since calcium plays a key role in muscle contraction, it has also been proposed as a mediator of translocation. [Holloszy, 1996 #19] While calcium has been shown to increase glucose transport in isolated muscle and blockade of calcium release from sarcoplasmic reticulum blocks glucose transport, calcium stimulated glucose transport does not share one a key characteristic of

exercise/ischemia mediated glucose uptake--it inhibits insulin induced glucose transport in contrast to exercise/ischemia induced glucose transport which is additive to insulin. [Draznin, 1987 #60; Lee, 1995 #61]

Several kinases have been proposed as mediators of exercise/contraction stimulation of glucose uptake. Inhibition of PKC by phorbol esters has been shown to decrease contraction stimulated glucose transport in skeletal muscle in vitro. [Cleland, 1989 #62] Exercise also activates the mitogen-activated protein kinase (MAP kinase). [Aronson, 1997 #64] But this mechanism is unlikely given recent that demonstration that pharmacological blockade of MAP kinase does not influence contraction stimulated glucose transport in vitro . [Hayashi, 1998 #50] Other kinases proposed to be involved in GLUT4 translocation include the c-Jun NH2 terminal kinase and p38 kinase. [Hayashi, 1997 #87] These kinases may mediate the effects of ischemia and AICAR in stimulating GLUT4 translocation. (figure 9)

Other changes that result from ischemia may also mediate the effects of ischemia Nitric oxide is released during skeletal muscle contraction and inhibition of nitric oxide synthase has been demonstrated to inhibit contraction mediated glucose transport but not insulin stimulated glucose transport. [Kapur, 1998 #57; Balon, 1997 #55] Other potential regulators of glucose transport include bradykinin [Kishi, 1998 #88] and kallikrein and adenosine. [Han, 1998 #56; Law, 1988 #58] While nitric oxide, bradykinin, and kallikrein are potential mediators of AICAR induced stimulation of glucose transport, it is unlikely since no potential mechanism whereby AICAR influences them has been identified. Since AICAR may increase local concentrations of adenosine and is structurally similar to adenosine, it is possible that its actions may be mediated by acting

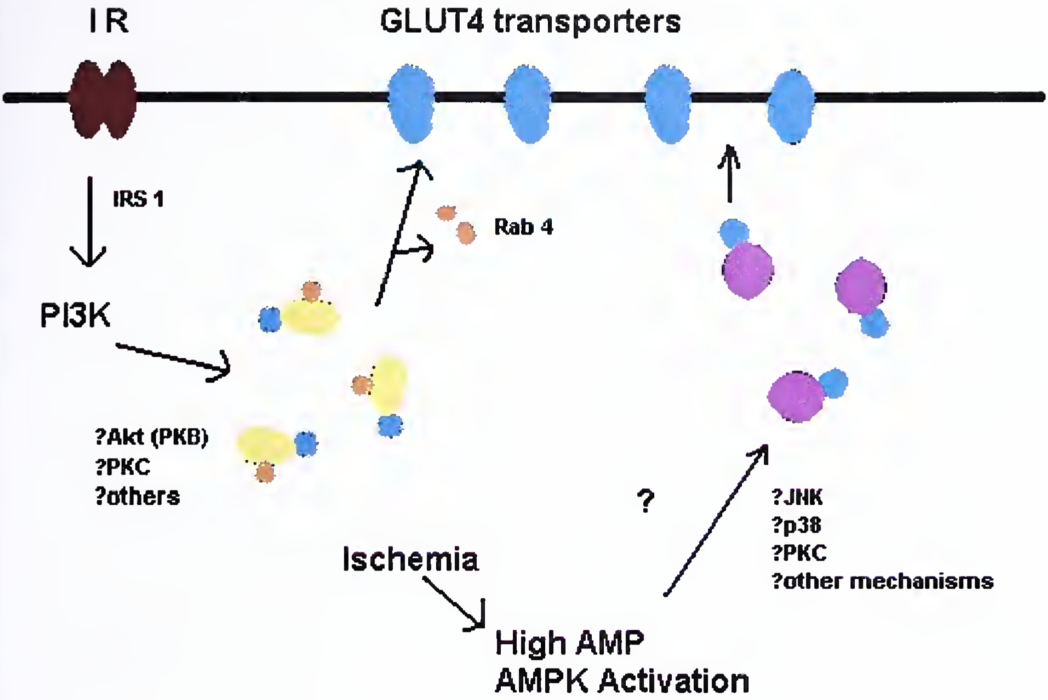


Figure 9 Schematic presentation of the potential mechanisms of insulin and ischemia induced GLUT4 translocation. Yellow circles are representations of insulin sensitive GLUT4 vesicles with specific makers such as Rab4 (orange). Pink circles represent ischemia and/or AMPK sensitive GLUT4 vesicles. IR is the insulin receptor. Blue represents GLUT4 transporters.

on adenosine receptors. However, AICAR is not known to act on adenosine receptors and inhibition of these receptors does not affect AICAR stimulation of glucose uptake. [Russell, 1998 #44]

Downstream Mechanisms of GLUT4 Translocation

The downstream mechanisms responsible for AMPK mediators are unclear. Studies from the insulin mediated transport indicate that there are many potential mechanisms by which AMPK may effect glucose translocation. GLUT4 cycles between the plasma membrane and intracellular compartments with most staying intracelluarly in the basal state. [Kandror, 1996 #67] Stimulation of glucose uptake can be effected either through an increase in the rate of GLUT4 vesicle exocytosis or by a decrease in endocytosis. [Stephens, 1995 #10] Insulin acts largely by stimulating exocytosis, causing a 10-20 fold increase in exocytosis compared to only a 2-3 fold decrease in the rate of endocytosis. GLUT1 is distributed more evenly between the intracellular compartment and the sarcolemma and plays a smaller role in altering glucose transport in response to extracellular signals. [Young, 1995 #79; Fischer, 1997 #21] Insulin causes a more modest stimulation of GLUT1 and exercise has been reported not to influence GLUT1 in skeletal muscle but we have previously shown that ischemia in myocardium, like AICAR, does cause translocation of GLUT1, though to a more modest degree. [Young, 1995 #79]

GLUT4 Vesicles

There is evidence which suggests that GLUT4 vesicles are heterogeneous. The additive nature of exercise and insulin mediated effects on glucose transport and GLUT4 translocation suggest there may be further or separate pools of GLUT4 vesicles that are more responsive to specific stimuli. [Douen, 1990 #65] Novel membrane fractionation

techniques in cultured adipocytes have identified distinct intracellular locations or pools of glucose transporters, one responsive to exercise and another more responsive to insulin. [Hayashi, 1997 #17; Coderre, 1995 #63] Rab4, a GTP binding protein involved in formation, targeting, and fusion of vesicles by acting as a molecular switch by interconversion.from a GDP-bound form to a GTP-bound form [Van der Sluijs, 1992 #89] is redistributed by insulin but not by exercise. [Hayashi, 1997 #17; Sherman, 1988 #90] It is possible that GLUT4 carrying vesicles have unique receptors making them more sensitive to particular stimuli.

Regardless of whether GLUT4 vesicles are segregated by responsiveness to stimuli or not, GLUT4 transporters require specific signals to be properly targeted for intracellular storage. [Pessin, 1999 #66] Recent studies suggest that the cytoplasmic C-terminus of GLUT4 contains a dileucine motif whose absence leads to accumulation of GLUT4 in the plasma membrane[Verhey, 1995 #82]. This sequence has been shown to be involved in internalization and while similar motifs responsible for sequestration have not been identified, morphologic and kinetic studies suggest they are present in the GLUT4 cytoplasmic C-terminus. [Pessin, 1999 #66]

GLUT4 Exocytosis

A recent review proposes two major, overlapping mechanisms whereby insulin and other stimimuli can regulate GLUT4 exocytosis . [Pessin, 1999 #66] The first, “retention model” (figure 10) suggest that GLUT4 vesicles participate in the constituitively recycling endosomal system with regulation of exocytosis by regulation of GLUT4 vesicles through specialized “retention receptors” that control their entry into the endosomal system. GLUT4 protein is localized in small vesicles and tubulovesicular

structures with lesser amounts in the trans-Golgi network, clathrin-coated vesicles and endosomes. [Rodnick, 1992 #68; Slot, 1991 #69] GLUT4 vesicles and tubulovesicular are found adjacent to endosomes underlying plasma membrane. [Pessin, 1999 #66] For insulin induced translocation, the insulin-responsive aminopeptidase (IRAP), also known as vp165, may act as a retention receptor. IRAP is known to probably co-localize with GLUT4 vesicles and displays some homology with the GLUT4 C-terminus. [Kandror, 1994 #73] In addition, introduction of the cytoplasmic domain of IRAP results in plasma membrane translocation of GLUT4. [Waters, 1997 #91] Despite the evidence supporting the role of the recycling endosomal system and sequestering, retention receptors, there are other findings which suggest that this model does not account for all the regulation of iGLUT4 trafficking. GLUT4 containing vesicles can be differentiated from the constituitively recycling endosomal system. GLUT4 vesicles are enriched in the v-SNARE (vesicle SNAP receptors) protein , VAMP2 but not the VAMP3 isoform present in the constituitively recycling endosome population. [Martin, 1998 #71] Furthermore, ablation of the endosomal population as tracked by the transferrin receptor suggest that insulin-stimulated GLUT4 translocation can occur independent of the endosomal system. This finding with insulin may also be true with ischemia.

Protein complexes in the GLUT4 vesicle compartment such as the v-SNARE VAMP2 suggest that a synaptic-like mechanism similar to that found in the vesicle trafficking regulation of neurotransmitters may regulate GLUT4 vesicle transport. [Pessin, 1999 #66] (figure 11) v-SNAREs pair with their matching receptor complexes at the target membrane (called t-SNAREs for target SNAP receptors). Interaction between v-SNAREs and t-SNAREs in combination with accessory proteins is responsible for

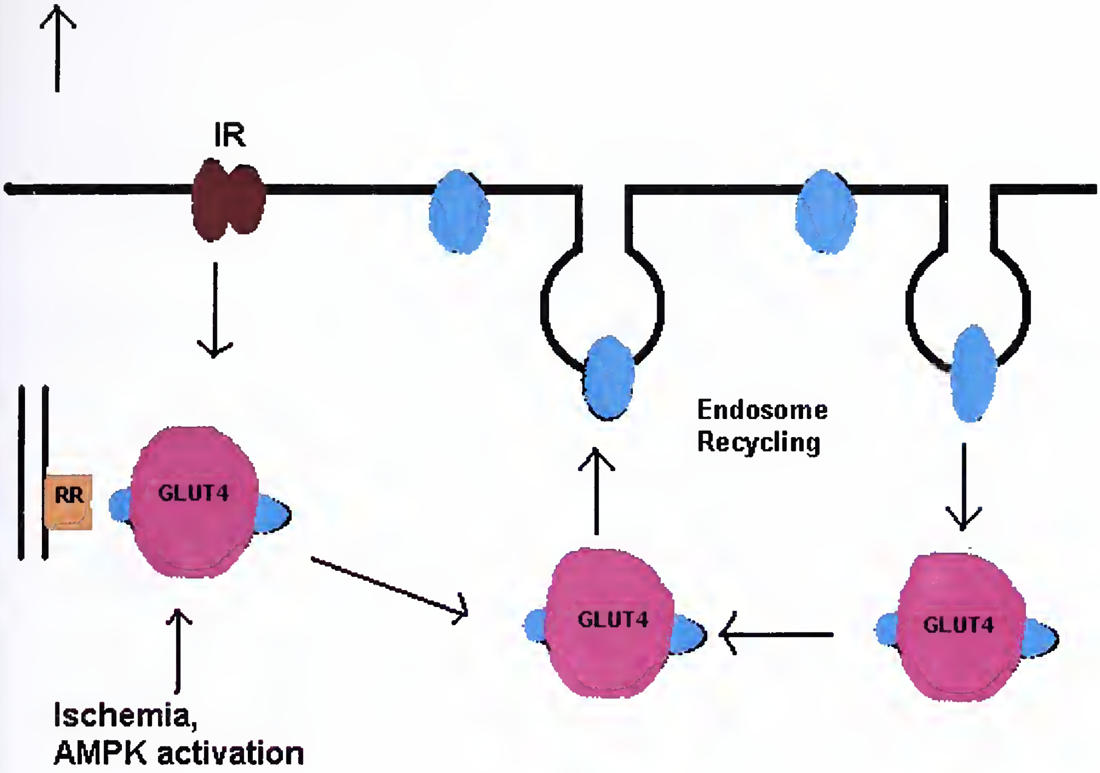


Figure 10. Schematic illustration of retention hypothesis of GLUT4 trafficking. GLUT 4 vesicles are sequestered by association with retention receptors (RR). Insulin or other stimuli such ischemia or AMPK activation can cause dissociation from RR and entry of

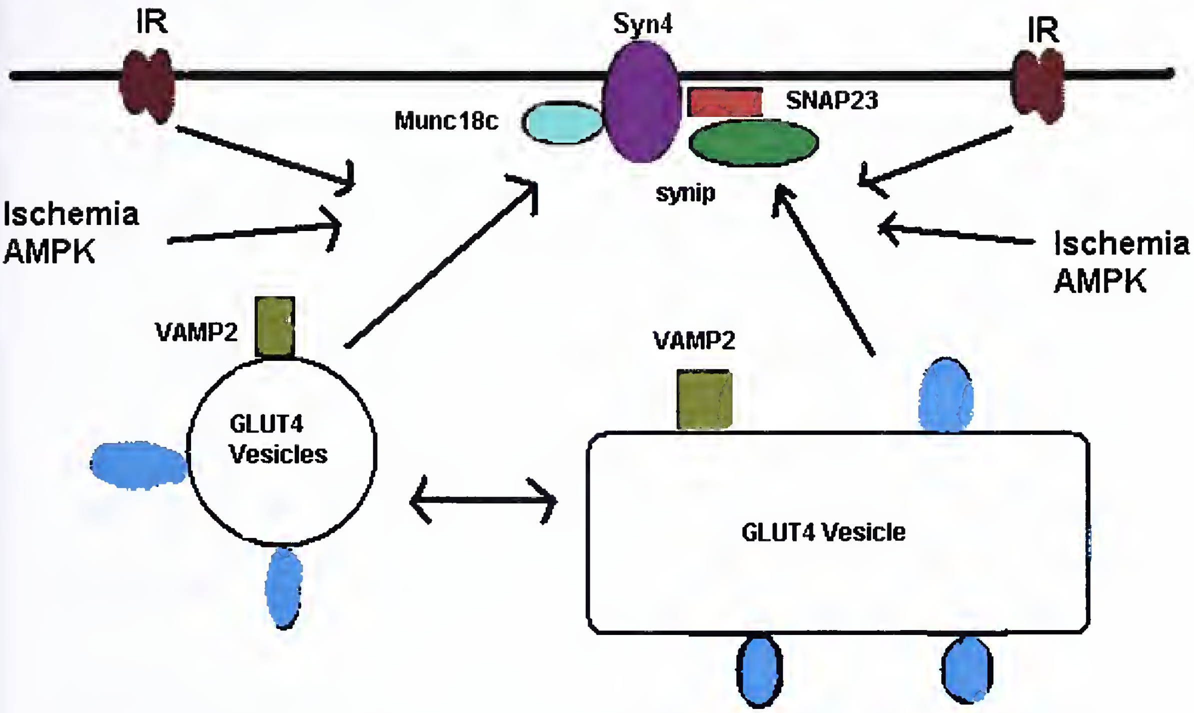


Figure 11. Schemtic presentation of the hypothesized synaptic model of GLUT4 trafficking. GLUT4 vesicles are localized to small synaptic like vesicles and larger tubulovesicular compartments in equilibrium. Both have the v-SNARE VAMP2. GLUT4 translocation can be induced by interaction of VAMP2 with t-SNARE complex. (Blue figures represent GLUT4. GLUT4 at plasma membrane not shown).

determining trafficking and formation of complexes required for membrane fusion [Pessin, 1999 #66] . VAMP2 has been identified on GLUT4 vesicles and may act as the v-SNARE protein while studies using blocking antibodies, dominant interfering mutants and peptide inhibitors suggest that several proteins (including syntaxin 4 and SNAP23/Syndet) act as the t-SNARE proteins. Munc18c and Synip have recently been shown to bind to syntaxin 4 and may be part of the complex that regulates the interaction with VAMP2. Binding of Munc18c to syntaxin 4 prevents its binding to VAMP2 and an increase in expression of Munc18c inhibits insulin-stimulated GLUT4 translocation. While the precise role of Synip has not been delineated, it interaction with syntaxin 4 is known to be regulated by insulin.

Role of G-Proteins and GLUT4 Endocytosis

There is a role for G-proteins in GLUT4 trafficking but the specific mechanisms have not been elucidated. GTPγS causes GLUT4 translocation [Pessin, 1999 #66] and G-protein coupled receptors linked to Gq can also induce GLUT4 translocation. [Kishi, 1998 #88] Signalling intermediates such as Grb2 involved in G-protein signal transduction are also known to interact with IRS1, a key intermediary of insulin stimulated translocation through PI3K. [Holman, 1997 #54]

G-proteins may have an especially significant role in GLUT4 endocytosis. In the basal state, GLUT4 in plasma membrane is in coated pit regions and disruption of clathrin has established a role for clathrin-mediated edocytosis in GLUT4 internalization. GTPase dynamins are essential to this process and in addition to GTPase sites have domains which interact with signaling intermediates such inositol phospholipids and amphiphysin. [Volchuk, 1998 #77] Dynamin may play an important role in regulating

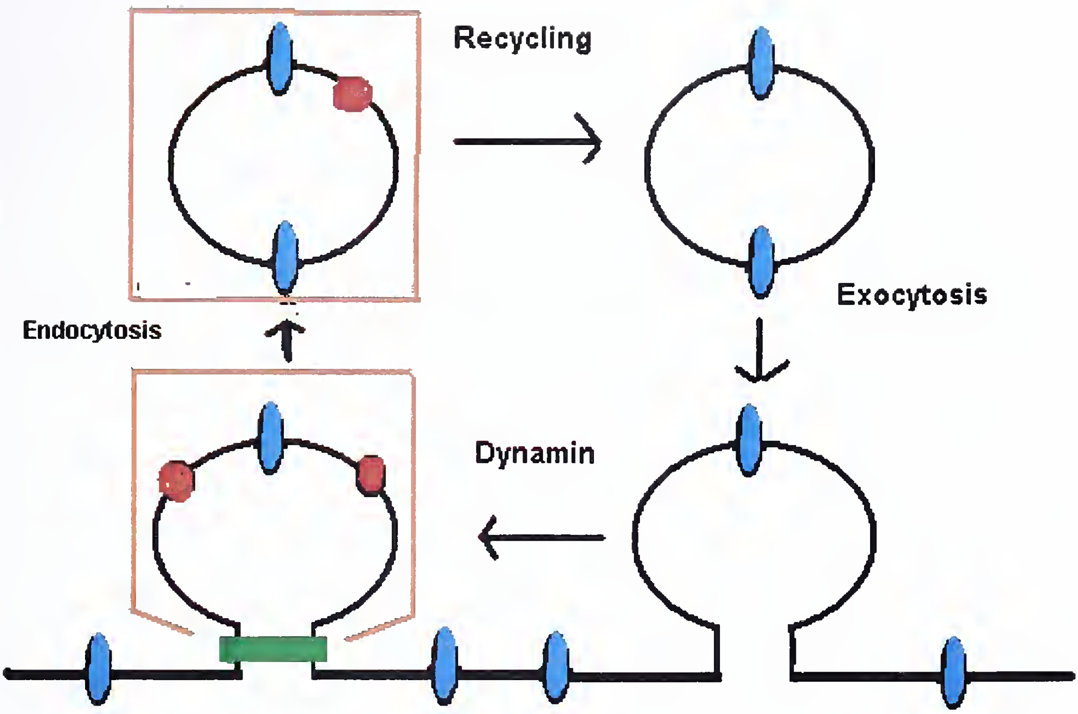


Figure 12. The internalization of GLUT4 occurs through a dynamin-dependent mechanism. After translocation, GLUT4 accumulates in clathrin-coated (orange) invaginations of the plasma membrane. This occurs through the interaction of of GLUT4 with complexes such as AP2 (red circle). Dynamin (green rectangle) is recruited to thesese structures and is required for the intracellular clathrin coated vesicles. These vesicles rapidly lose their coat structure and are available for translocation or to repopulate the intracellular pool of GLUT4 vesicles.

GLUT4 endocytosis and may serve as a target of AMPK phosphorylation. (figure 12) Insulin has been reported to induce tyrosine phosphorylation of dynamin [Baron, 1998 #75]and microinjection of a dynamin peptide encompassing anphiphysin binding sites prevents GLUT4 edocytosis and cause accumulation in the sarcolemma. [Volchuk, 1998 #77]Expression of a dominant interfering mutant of dynamin has also been shown to prevent GLUT4. [Al-Hasani, 1998 #76] This GTPase may thus be a target for AMPK phosphorylation.

Fate of glucose

While AICAR infusion increased myocardial glucose uptake, our findings do not define the fate of the glucose. Once in the cell as G6P there are four potential fates of glucose: glycogen synthesis, glycolysis, glucose oxidation or the pentose phosphate shunt. G6P concentrations were unchanged in the AICAR regions, indicating that any increase in glucose conversion to G6P was balanaced by increased distal metabolism. Although AMPK inhibits glycogen synthase and stimulates glycogen phosphorylase in liver effectively decreasing synthesis and increasing breakdown, [Gillespie, 1992 #92] it does not affect glycogen synthase in musle but still stimulates glycogen phosphorylase leading to increased glycogenolysis. [Young, 1996 #93] However, in the rat, intravenous AICAR infusion does no affec skeletal muscle glycogen. [Bergeron, 1998 #86] But in myocardium it appearst to increase muscle glycogen. [Russell, 1998 #44] The latter effect may be due to preferential flux of glucose into glycogen which may occur due to the increase in arterial lactate concentrations accompanying AICAR infusion. [Russell, 1998 #44] We saw a fall in arterial lactate suggesting that the glucose was not released as an end product of glycolysis.

Oxygen uptake was not stimulated in the AICAR region. Increased glucose oxidation could be expected to stimulate oxygen uptake, especially in light of the enhanced FFA uptake that is also likely to have been oxidized (see below). The energy content of the cell as measured by creating phosphate and ATP did not change. These findings suggests that AICAR did not stimulate glucose oxidation. A potential fate of glucose is to enter the pentose phosphate shunt to provide NADPH for reductive biosynthesis. The activity of this pathway is very low in muscle [Stryer, 1995 #84] and unlikely to be the primary fate of the additional glucose that is taken up. It is important to note that if the glucose did not become metabolized through a single pathway, the measurement techniques we used may have been inadequate to record several small changes.

Effect on FFA

Our study found a small increase in FFA uptake following AICAR infusion. This is consistent with previous studies which showed a stimulation of FFA entry into mitochondria due to an increase in CPT1 activity caused by a decrease in malonyl-CoA levels which resulted from the phophorylation of ACC and a 50% increase in FFA oxidation. [Kudo, 1995 #36] While we did not measure FFA oxidation or triglyceride synthesis it is likely that the additional FFA was oxidized through the phosphorylative inhibition of ACC. However, we did not see an increase in oxygen uptake.

Hemodynamics and Functional Effects

While there was a trend for higher blood flow in the AICAR infused region, the overall myocardial contractility and cardiac output were significantly depressed following region infusion of AICAR. Though much lower rates of systemic AICAR

infusion were used, earlier investigators had found that AICAR had no functional effects on non-ischemic myocardium [Young, 1991 #94; Gruber, 1989 #95] and improved cardiac function in ischemic myocardium. [Young, 1991 #94; Gruber, 1989 #95]

Clinical studies also show a beneficial effect of AICAR on myocardial function. A meta-analysis of 5 international randomized trials showed that intravenous infusion of AICAR (0.1mg/kg/min) following surgery reduced perioperative myocardial infarction by 27% and decreased the incidence of cardiac death by 50% in the postoperative period. [Mangano, 1997 #96] Another study found that AICAR improved ventricular function in pacing-induced ishcmia. [de Jonge, 1997 #97]

The mechanism for any functional effect of AICAR in ischemic myocardium is not known but a role for increases in local concentrations of adenosine has been postulated. [Gruber, 1989 #95] Despite the structural homology of AICAR with adenosine, AICAR is not known to act on adenosine receptors and we did not measure tissue or venous concentrations of adenosine. However, the functional effects that we found are consistent with the known vasodilatory and negative inotropic effects of adenosine receptor stimulation in myocardium. [Shryock, 1997 #98] Other investigators [Gruber, 1989 #95] had not found an increase in adenosine concentrations in non-ischemic tissues.

Limitations of Study and Future Directions

The functional findings are limited by a lack of control animal experiments to separate the potentially confounding effects of infusion and additional operative time under anesthesia with open chest. While it was not a major aim of our study to investigate these effects, additional studies with saline infused controls would clarify

these findings. The control used in this study (LAD vs LCX) is not ideal because the two arterial beds are not completely separated and communicate via collaterals. Moreover, the extended period of study may allow AICAR to accumulate and cause systemic effects. However the concentration of AICAR in the LAD (AICAR) bed was more than seven fold higher than in the LCX (control) bed and more than 20-fold higher than in the systemic circulation. The control bed was also not infused with saline and this may also be a confounding variable. However, the rate of infusion is about 0.5% of the normal canine coronary blood flow (0.25ml/min vs. 50ml/min) and unlikely to cause significant disturbances in flow parameters.

The link between AMPK activation and glucose transporter translocation cannot be unambiguously established. We verified that AICAR infusion did lead to a concentration of ZMP that is known to cause significant activation of AMPK and that the enzyme was stimulated by infusion. However, while AICAR is known to activate AMPK and has not been shown to cause any other effects on the cellular energy state, it is possible that it has unidentified effects which may confound our findings. Experiments with inhibitors of AMPK such as the ser-thr kinase inhibitor 5-iodotuberidon or genetic techniques to suppress or overexpress the AMPK gene in myocardium would provide further support for a role of AMPK in ischemia mediated changes in glucose transport. This study provides the background necessary to pursue these investigations.

Conclusion

This study suggests that metabolic stress such as ischemia stimulate glucose uptake by translocation of GLUT4 and GLUT1 through a mechanism that involves AMPK. Additional studies are necessary to delineate the downstream effectors that lead

to translocation. This system may also mediate other the cellular and tissue responses to ischemia. Augmented stimulation of the system by AICAR or other potential stimulators may enhance myocardial function during acute episodes of ischemia or in the long term, may allow improvement of function by altering the metabolic milieu or other potential mechanisms.

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